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Our studies on transcriptional regulation of the c-*myc* proto-oncogen are divided into two subprojects: i) studies on Blimp-1, a transcriptional repressor of c-*myc* and ii) studies to determine the mechanism by which estrogen induces c-*myc* transcription in estrogen-dependent breast cancer cells. Blimp-1 was initially identified as a master-regulator of B-lymphocyte differentiation and was thought to be B-cell specific. We recently showed that Blimp-1 is a site specific repressor of c-*myc* transcription. Our studies reported here provide evidence that Blimp-1 plays a role in terminal differentiation of many cell lineages. We show that Blimp-1 mRNA is induced upon terminal differentiation of promyelocytic cells in culture and that Blimp-1 mRNA is present in many adult tissues. We have also demonstrated the ability of Blimp-1 to repress growth of MCF-7 breast cancer cells, which depend upon c-Myc activity for their transformed growth. In the second project, runon transcription has been used to show that estrogen induces transcription initiation but does not alter polymerase processivity on the c-*myc* gene in MCF-7 cells. We have identified four DNaseI hypersensitivity sites in the c-*myc* gene which are induced in response to estrogen. They are good candidates to be unusual estrogen response elements.

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Katherine Celano 10/27/97

PI - Signature Date

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(Since this work is divided into two subprojects; separate reports are presented.)

## SUBPROJECT I: BLIMP-1, A REPRESSOR OF C-MYC TRANSCRIPTION, IS EXPRESSED IN MULTIPLE LINEAGES AND MAY BE A TUMOR SUPPRESSOR

### INTRODUCTION

**Blimp-1 is a "master regulator" of B-cell terminal differentiation.** Blimp-1 (B lymphocyte induced maturation protein) was identified in a subtractive screen as a protein which is induced upon stimulation of the BCL1 B-cell lymphoma line with IL-2 + IL-5 (1), a treatment which induces terminal differentiation. Blimp-1 mRNA was detected by Northern analyses in that study only in spleen, B-cell lymphomas and plasmacytomas (1), a pattern consistent with a role in terminal B-cell differentiation. Ectopic expression of Blimp-1 in BCL1 cells can drive B-cell terminal differentiation as evidenced by J-chain transcription, immunoglobulin secretion and expression of Syndecan on the cell surface (1). Sequence analysis of Blimp-1 cDNA revealed that the protein contained five zinc fingers, strongly suggesting that it was a DNA-binding protein, possibly a transcription factor (1). Such a function would be very consistent with its role as a master regulator of terminal differentiation because one might imagine that Blimp-1 would control the expression of an array of genes which would be sufficient to initiate terminal differentiation. However, the initial report did not identify a DNA binding sequence for Blimp-1 or any genes which were direct targets of Blimp-1.

The role of Blimp-1 in B-cell development has been studied further. Blimp-1 expression in B cells was found to correlate with Ig secretion and it was suggested that Blimp-1 may play a role in the B-cells' choice between a memory or plasma cell fate (2). The polyclonal mitogen LPS induces Ig secretion, Blimp-1 expression and B-cell differentiation of splenic B cells (2). IL-5 and the IL-5 receptor appear to be important for inducing Blimp-1 in B cells (3) and ectopic expression of the early B cell transcription protein BSAP blocks B cell differentiation, Ig secretion and Blimp-1 expression (4). Although Blimp-1 appears to activate J-chain transcription, the effect may be indirect since no Blimp-1 binding site or response element has been found in the J chain gene (M. Koshland, pers. commumication).

**Blimp-1 represses c-*myc* gene transcription.** Our group previously identified a plasmacytoma-specific protein which bound to a site located 290 bp 5' of the c-*myc* P1 initiation site (5, 6). Because of its occurrence only in plasmacytomas, we hypothesized that the protein was a repressor of c-*myc* transcription. Note that in plasmacytomas one c-*myc* allele is invariably translocated to the IgH locus. The translocation in these tumors usually occurs between c-*myc* exons 1 and 2, removing P1 and P2 and the regulatory region 5' of them (including the -290bp site). This causes elevated transcription of the translocated allele of c-*myc* from cryptic promoters activated by IgH elements. The resulting deregulated levels of c-Myc are one causal step in malignant transformation of plasmacytomas (7). However, the normal, non-translocated c-*myc* allele in plasmacyotmas is transcriptionally silent, presumably representing the status of the c-*myc* gene in normal plasma cells which are terminally differentiated, non-dividing cells and would not be expected to express c-*myc*. Site-directed mutation of the -290 binding site confirmed that the site was bound by a transcriptional repressor in plasmacytomas and the protein was called Plasmacytoma Repressor Factor (PRF) (8).

Based on the observation that the sequence of the PRF site was similar to that of PRD1-BF1 (the only known binding site for Blimp-1), we suspected that Blimp-1 might encode PRF. The following experimental evidence was used to confirmed this hypothesis: 1) demonstration that recombinant Blimp-1 binds the PRF site at -290bp in the c-*myc* gene, 2) demonstration that antiserum to recombinant Blimp-1 ablates site-specific binding of endogenous PRF in plasmacytoma extracts to the -290 site of the c-*myc* gene and 3) demonstration that cotransfected Blimp-1 represses the c-*myc* promoter in a PRF-site dependent manner (9). In addition, we showed that ectopic expression of Blimp-1 led to decreased levels of endogenous c-Myc in both 18-81 preB and BCL1 lymphoma cells (9). In the A-MuLv-transformed preB cell line 18-81, ectopic expression of Blimp-1 caused apoptosis, presumably because expression of the v-Abl oncogene blocks further differentiation by repressing expression of RAG1 and RAG2 and blocking activation of NF-κB (10, 11). Blimp-1-dependent apoptosis of 18-81 cells was overcome by ectopic expression of c-Myc, providing

additional evidence that c-*myc* is an important target of Blimp-1 (9). No other direct target gene for the Blimp-1 repressor has been identified in B cells. Although increased J chain transcription correlates with Blimp-1 expression, no Blimp-1 binding site has been identified in the J-chain gene (M. Koshland, pers. comm.). Thus, our studies identified PRF as Blimp-1 and identified c-*myc* as a functionally important target gene repressed by Blimp-1 in B cells.

Only a few "master regulators" which are capable of initiating an entire developmental program have been identified in mammalian cells. Blimp-1 is the first of these "master regulators" shown to regulate the c-*myc* gene. This finding is consistent with previous studies showing that expression of c-*myc* blocks terminal differentiation (12-14) and with the identification of Blimp-1 as a protein which causes terminal B-cell differentiation (1). Our demonstration that Blimp-1 represses c-*myc* as part of a program of terminal differentiation makes it possible, for the first time, to study an aspect of c-*myc* regulation which is directly associated with a developmental decision to halt proliferation and to proceed with terminal differentiation.

**Blimp-1 is a good candidate for a tumor suppressor.** Since elevated and deregulated expression of c-Myc is a causal event in malignant transformation of many tumors (15, 16), proteins such as Blimp-1 which repress c-Myc transcription might function as tumor suppressors. It is striking that recent gene mapping data strongly support this suggestion. Blimp-1 maps to human chromosome 6q21-22.1 and to the syntenic region on murine chromosome 10 (17). Various regions of human chromosome 6q, including 6q21-22, are deleted in a wide variety of tumors, leading to the suggestion that tumor suppressor gene(s) are located within this region.

**Breast cancer** is associated with deletions at multiple chromosomal regions, including 6q. More detailed mapping identified one of three regions of deletion in breast cancer at 6q16.3-q23 (18, 19). Microcell experiments in which portions of chromosome 6 were used to suppress a tumorigenic phenotype in breast cancer cell lines identified 6q21-q23, the region including *blimp-1*, as one region encoding a tumor suppressor (20).

Many other cancers also have 6q deletions which within a region that appears to include Blimp-1. Deletions of chromosome 6q have been reported in **acute and chronic lymphocytic leukemia** (ALL and CLL) and prolymphocytic leukemia (PLL), and have been associated with t(14;18), which involves translocation of the *bcl-2* gene, in non-Hodgkin's lymphoma (NHL) (21, 22). Several regions on chromosome 6q are deleted in these tumors: one includes the region of the Blimp-1 gene located at 6q21-6q22.1. The most refined mapping reported recently (23) defined a 2 Mb region of 6q21, between D6S447 and D6S246, which was deleted in 32 lymphoid malignancies with 6q deletions. *Blimp-1* is closely linked to D6S447 and appears to be located between D6S447 and D6S268 (17). Since these ESTs have been ordered: Cen DS6447-DS6268-DS6278-DS6246 (24) it appears that the *Blimp-1* gene is within the deleted region. Therefore *Blimp-1* is a likely candidate for the tumor suppressor gene which is deleted in lymphoid malignancies harboring deletions in the 6q21 region. Deletions in 6q are found in sporadic and familial **melanoma** and it is assumed that tumor suppressor genes lie within the deleted regions (25). Loss of heterozygozity involving 6q were found in 31% of melanomas in one study (26) and 35% in another (27) and 6q21 is a frequently deleted region in melanomas (28). Chromosome 6 can reverse the transformed phenotype of melanoma cell line UACC903 (29). Deletions in **endometrial tumors** were mapped to three regions of 6q, one located at 6q21-23. (30). A comprehensive analysis of 6q deletions in **prostate cancer** showed that 6q14-21 may harbor a tumor suppressor gene important in prostate carcinogenesis (31). Deletion of 6q21-22-->qter was shown to be a consistent structural cytogenetic abnormality in **gastric carcinomas** (32). Finally, 6q21 is among several chromosomal regions involved in rearrangements in ovarian cancer (33) and a gene on 6q14-21 restored senescence to immortal **ovarian tumor** cells, confirming the location of a senescence gene in this region (34).

In the present study we have addressed two questions. First, is Blimp-1 expressed outside of the B-cell lineage? The original report of Blimp-1 characterized it as a B-cell specific protein. However, we show that it is expressed in many non-B cell and non-hematopoietic lineages and that it is induced upon terminal differentiation of promyelocytic cells. Secondly, we have asked if ectopic expression of Blimp-1 might alter the growth phenotype of breast cancer cell lines where the transformed phenotype depends on the presence of c-Myc. Our preliminary results suggest that induction of Blimp-1 expression alters the growth of MCF-7 cells in culture.

## BODY

METHODS

**Cell Culture.** HL-60 promyelocytic and U937 promonocytic cell lines were cultured in RPMI supplemented with 10% fetal calf serum. To initiate macrophage differentiation, HL-60 or U937 cells were supplemented with phorbol myristic acid (PMA). Using flow cytometry we monitored Mac-1(CD11b), ICAM and Class II MHC, which are expressed during macrophage differentiation. Differentiation was also monitored by staining for nonspecific and specific esterase activity (35). Granulocytic differentiation of HL-60 cells was initiated by culturing the cells in DMSO. It was monitored by NBT (Nitroblue Tetrazolium) which is converted to insoluble intracellular blue formazan by superoxide (36). MCF-7 cells were grown under + estrogen conditions in IMDM supplemented with 10μg/ml of insulin and 10% fetal bovine serum (FBS). To estrogen-deplete the cells, plates were washed once with PBS at 70% confluency and growth-arrested using phenol red-free IMDM supplemented with 10% charcoal stripped FBS for 48 hours.

**Northern Blots. Northerns.** Total RNA was prepared by lysing cells with 4 M guanidium thiocynate, 25mM sodium citrate, 0.5% n-lauryl sarcosine, 100mM mecaptoethanol. Lysates were pelleted through a 5.7 M CsCl cushion by centrifuging for 12 hr at 36,000 rpm in a SW50 rotor. For northern blots, 20-40 μg of total RNA was resuspended in 1X formaldehyde gel-buffer (0.1M MOPS pH 7.0, 40 mM sodium acetate, 5mM EDTA), 17.5% formaldehyde, 50% formamide, 1X gel-loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and separated on a formaldehyde-agarose gel (1% agarose, 1X formaldehyde gel buffer, 2.2 M formaldehyde). RNA was transferred to Hybond-N nylon membranes (Amersham, Arlington Hieghts, IL) by capillary action using 20X SSC (3M NaCl, 0.3M Sodium Citrate). RNA was fixed to the membrane by baking for 2 hr at 80°C and hybridized with radiolabeled probes at 65°C in 1.5X SSPE (0.23 M NaCl, 1.5 X $10^{-2}$M $NaH_2PO_4$, 1.5 X $10^{-3}$M EDTA), 10% Polyethelene glycol, 7% SDS and 0.1 mg/ml salmon sperm DNA for at least 12 hours. Filters were washed once at 22°C in 2X SSC, 0.1% SDS for 15 min, once at 65°C in 1X SSC for 15 min, 0.1% SDS and once at 65°C in 0.3X SSC, 0.1% SDS for 15 min. Filters were stripped by washing at 100°C in 0.1% SDS. Radiolabeled probes with a specific activity of $> 2 \times 10^8$ were generated by random primering gel isolated DNA fragments according to the manufacturer's specifications (United States Biochemical, Cleveland, OH). The membranes were hybridized sequentially with radiolabeled cDNA corresponding to Blimp-1, c-*myc* and GAPDH. The results were quantitated using a PhosphoImager and normalized to GAPDH.

RESULTS

### Blimp-1 is induced upon differentiation of two promyelocytic cell lines.

Since all terminally differentiated cells stop dividing, and therefore presumably need to shut off their expression of c-*myc*, we wondered if Blimp-1, a repressor of c-*myc* transcription during terminal differentiation of B cells, might play a similar role in cells outside of the B lymphocyte lineage. Therefore, we tested the hypothesis that Blimp-1 is induced upon terminal differentiation of other, non-B, cell lineages. Our initial experiments have utilized two well-established systems for terminal differentiation of cultured cells: i) HL-60, a multi-potential promyelocytic line which differentiates into macrophage-like cells in response to PMA and into granulocyte-like cells in response to DMSO and ii) U937, a promonoblastic line which differentiates into macrophages in response to PMA.

We observe clear and striking induction of Blimp-1 mRNA which accompanies all three differentiation programs studied--differentiation of U937 into macrophages, of HL-60 into macrophages and of HL-60 into granulocytes. Northern blots of total RNA prepared at intervals during the differentiation process were probed first with Blimp-1 cDNA, then stripped and probed with c-*myc* cDNA and finally stripped and probed with GAPDH cDNA. Fig. 1 shows a Northern blot of total RNA from HL-60 cells following activation with PMA. After 72 hrs. macrophage

differentiation was complete as judged by altered cell morphology and increased expression of Mac-1 and ICAM. During differentiation Blimp-1 mRNA was induced, showing one peak early and one peak late in the differentiation process. c-*Myc* mRNA, consistent with previous reports (15), decreased after an early increase. Although the precise patterns vary somewhat, we also observed similar patterns of increases in Blimp-1 mRNA and decreases in c-*myc* mRNA during differentiation of HL-60 cells to granulocytes in response to DMSO (Fig. 2) and of U937 cells to macrophages in response to PMA (Fig. 3). The data were quantitated by PhosphoImager and normalized for loading differences using GAPDH (Fig. 4). In each case, a decrease in c-*myc* mRNA correlates with a rapid increase in Blimp-1, consistent with the idea that Blimp-1 represses c-*myc* transcription in these cells. We do not yet know the significance of the prominent second increase in Blimp-1 mRNA.

**Blimp-1 mRNA Is Present in Non-Lymphoid Tissues.**

The finding that Blimp-1 mRNA is induced upon terminal differentiation of two promyelocytic cell lines in culture prompted us to probe more carefully the possibility that Blimp-1 mRNA may be expressed in some/many non-lymphoid adult tissues, especially tissues which contain terminally differentiated cells. Our initial analyses have been Northern blots. As shown in Fig.5, Blimp-1 mRNA is significantly lower in all normal tissues than in plasmacytoma lines such as P3X. Nevertheless, we find expression of Blimp-1 mRNA in many tissues. Highest levels are observed in lung and ovary. Heart, brain, skeletal muscle, small interstine, kidney and spleen also express Blimp-1 mRNA. Three sizes of Blimp-1 mRNA, 5.7, 4.0 and 3.4 kb are present in P3X cells; varying relative amounts of these three isoforms occur in different tissues. At present we do not know if these isoforms encode functionally different forms of the Blimp-1 protein; our cDNA is derived from the largest mRNA. Blimp-1 mRNA was undetectable in liver and thymus, as well as in 18-81 preB cells. These results clearly show that Blimp-1 expression is not limited to the B lymphocyte lineage but occurs in multiple tissues.

**Induction of Blimp-1 Expression Alters the Growth Rate of MCF-7 Breast Cancer Cells.**

Previous antisense studies have suggested that elevated levels of c-Myc are required for the transformed growth properties of MCF-7 breast cancer cells in culture.(37). Furthermore, microcell experiments in which a portion of human chromosome 6 which includes the *blimp-1* gene was introduced into MCF-7 cells suppressed their tumorigenicity (20). Thus, we hypothesized that ectopic expression of Blimp-1 in MCF-7 might be sufficient to alter the growth properties of the cells by repressing c-*myc* transcription. MCF-7 cells were stably transfected with an expression plasmid in which transcription of Blimp-1 cDNA was regulated by a metallothionein promoter. Transfectants containing the Blimp-1 plasmid, and mock transfected controls were then treated with cadmium to induce Blimp-1 expression. The results are shown in Fig. 5. Even prior to induction, the Blimp-1 transfectants grew more slowly than controls. This is likely to reflect the fact that some Blimp-1 is expressed prior to heavy metal treatment. Although cadmium treatment caused a modest decrease in the growth rate of control cells, it had a more significant effect on the Blimp-1 transfectants. These data are consistent with the possibility that ectopic expression of Blimp-1 is sufficient to decrease the growth of MCF-7 cells. We are currently determining the expression levels of both Blimp-1 and c-*myc* mRNA in these cells to determine if the molecular basis for the growth repressive effect involves repression of c-*myc* transcription.

DISCUSSION

Blimp-1 was originally identfied as a gene whose expression was induced upon terminal differentiation of B cells and its expression was thought to be limited to the B lymphocyte lineage (1). However, following the discovery that Blimp-1 represses c-*myc* transcription (9), we reasoned that many/most cells might express Blimp-1 since c-*myc* transcription is repressed whenever cells cease proliferation (15). We furthermore reasoned that Blimp-1-dependent repression of c-*myc* transcription might be involved in terminal differentiation in other cell lineages. To test this hypothesis, we have studied Blimp-1 mRNA expression in three models for myeloid cell differentiation in culture using promyeloid HL-60 and promonocytic U937 cells. Our data clearly

show that Blimp-1 mRNA is induced upon macrophage-like differentiation of U937 cells in response to PMA treatment, upon macrophage-like differentiation of HL-60 cells in response to PMA treatment and upon granulocyte-like differentiation of HL-60 cells upon DMSO treatment. Thus, our data provide the first evidence that Blimp-1 may play a role in the differentiation program of cells outside the B lymphocyte lineage and suggest the possibility that Blimp-1 may be important in terminal differentiation of many cell lineages.

Our results are also consistent with the possibility that Blimp-1 acts to repress c-*myc* transcription in HL-60 and U937 cells since there is a reasonably good correlation between an increase in Blimp-1 mRNA and a decrease in c-*myc* mRNA. However, further experiments will be necessary to establish a clear causal relationship between induction of Blimp-1 mRNA and the decrease in c-*myc* mRNA in HL-60 and U937 cells. It is interesting to note, however, that previous studies have shown that blocking c-*myc* using anti-sense nucleotides is sufficient to drive terminal differentiation of HL-60 cells (38) and blocking c-Myc or Max triggered terminal myeloid differentiation (51), suggesting that the c-*myc* gene might be a (or the) primary target for Blimp-1 during myeloid differentiation.

Knowing that expression of Blimp-1 was not limited to B lymphocytes, we have analyzed its expression pattern in major adult tissues. Our data show that Blimp-1 is expressed not only in spleen, but also in heart, brain, ovary and lung. This expression pattern clearly demonstrates that Blimp-1 is not solely a "B-cell specific" protein and provides additional support for the hypothesis that Blimp-1 may be an important player in terminal differentiation of many tissues. Blimp-1 mRNA levels in all normal tissues were significantly lower than those observed in plasmacytoma cell lines. This may reflect the fact that normal tissues contain a mixture of cells at different developmental stages, only some of which express Blimp-1. Alternatively or in addition, plasmacytomas may express abnormally high levels of Blimp-1. The hypothesis that Blimp-1 is only expressed in cells within a tissue which have reached a certain stage of differentiation will be tested by performing *in situ* hybridization experiments to determine the amount of Blimp-1 mRNA in individual cells within different tissues. Both heart and brain contain terminally differentiated cells which have lost the ability to divide. In mice, cardiac myocytes lose their capacity to divide soon after birth and c-*myc* mRNA levels decrease from day 13 of embryonic development in those cells (39). Thus, the presence of Blimp-1 mRNA in these tissues provides support for the hypothesis that Blimp-1 is important for repression of c-*myc* during terminal differentiation of many cell lineages. Absence of Blimp-1 mRNA in liver and thymus is intriguing since liver cells and thymic T lymphocytes retain their proliferative capacity. This suggests the interesting possibility that Blimp-1 expression correlates with commitment to cease proliferation.

Given the importance of c-Myc expression for determining the transformed phenotype of many tumors including breast cancers, Blimp-1, as a repressor of c-*myc* transcription, might have the ability to alter the growth or transformed phenotype of tumor lines. Since previous studies have shown that MCF-7 cells have altered growth patterns when c-Myc is inhibited using antisense technology (37), we are testing the effect of ectopic expression of Blimp-1 in MCF-7 cells. Our current results are preliminary and more data will be necessary before we can draw firm conclusions. However, identification of two transfected lines where induction of Blimp-1 expression leads to significantly decreased doubling times is encouraging. If subsequent studies confirm our preliminary results, it will provide additional support for the critical importance of c-Myc in determing malignancy in breast cancers. Furthermore, such data will strengthen the possibility that Blimp-1 may function as a classical tumor suppressor in breast or other cancers where deletions on chromosome 6 in the region containing *blimp*-1 have been observed.

FIGURE LEGENDS

**Fig. 1A. Blimp-1 mRNA is induced when HL-60 cells differentiate in reponse to treatment with PMA.** Total RNA was prepared from HL-60 cells at the times indicated following treatment with PMA. The top panel shows a Northern blot with 20 μg/lane of total RNA probed with Blimp-1 cDNA; the lower panel shows the same blot after stripping and reprobing with c-*myc* cDNA.

**Fig. 1B. Blimp-1 mRNA is induced when HL-60 cells differentiate in reponse to treatment with DMSO.** Total RNA was prepared from HL-60 cells at the times indicated following treatment with DMSO. The top panel shows a Northern blot with 20 μg/lane of total RNA

probed with Blimp-1 cDNA; the lower panel shows the same blot after stripping and reprobing with c-*myc* cDNA.

**Fig. 1C. Blimp-1 mRNA is induced when U937 cells differentiate in reponse to treatment with PMA.** Total RNA was prepared from U937 cells at the times indicated following treatment with PMA. The top panel shows a Northern blot with 20 μg/lane of total RNA probed with Blimp-1 cDNA; the lower panel shows the same blot after stripping and reprobing with c-*myc* cDNA.

**Fig. 2. Graphic Representation of Blimp-1 and c-*myc* mRNA Levels During Differentiation of Promyelocytic Cell Lines.** These data were derived from Northern blots which were hybridized sequentially with probes corresponding to Blimp-1, c-*myc* and GAPDH cDNAs. The bands were quantitated using a PhosphoImager and normalized to GAPDH. They are expressed relative to the highest level, taken as 100%.

**Fig. 3. Expression of Blimp-1 mRNA in Adult Murine Tissues.** Total RNA (20 μg) from adult tissues was analyzed by Northern blotting using a Blimp-1 probe and compared to the levels in an equivalent amount of RNA from plasmacytoma P3X.

**Fig. 4. Growth Rate of MCF-7 Transfectants Expression Ectopic Blimp-1.** A. MCF-7 cells were stably transfected with an empty vector control (dashed lines) or with an expression vector in which Blimp-1 cDNA was regulated by the sheep metallothionein promoter(solid lines). The growth rate of these cells in the presence and absence of cadmium is shown. B. Northern blot probed with Blimp-1 cDNA showing induction of ectopic Blimp-1 upon treatment of MCF-7 transfectant clone with cadmium.

**Fig. 1**

## A. HL-60 Cells Treated with PMA

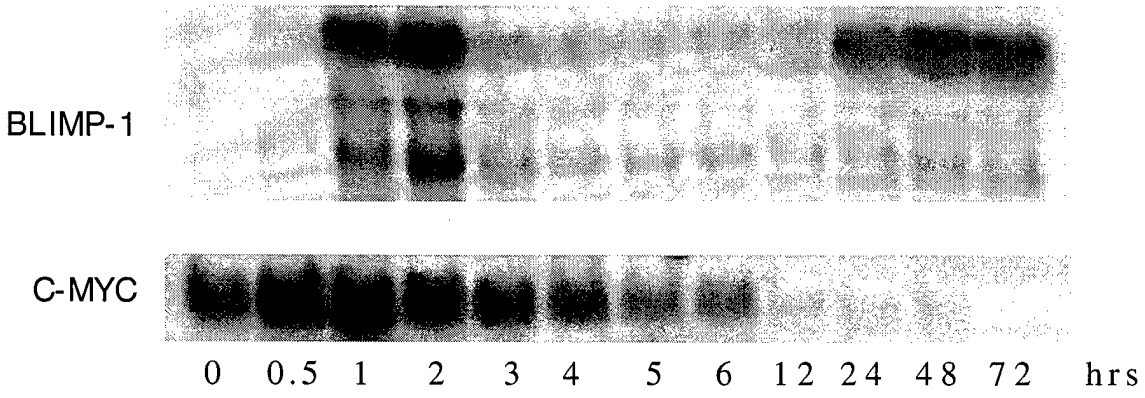


Fig. 1

**B. HL-60 Cells Treated with DMSO**

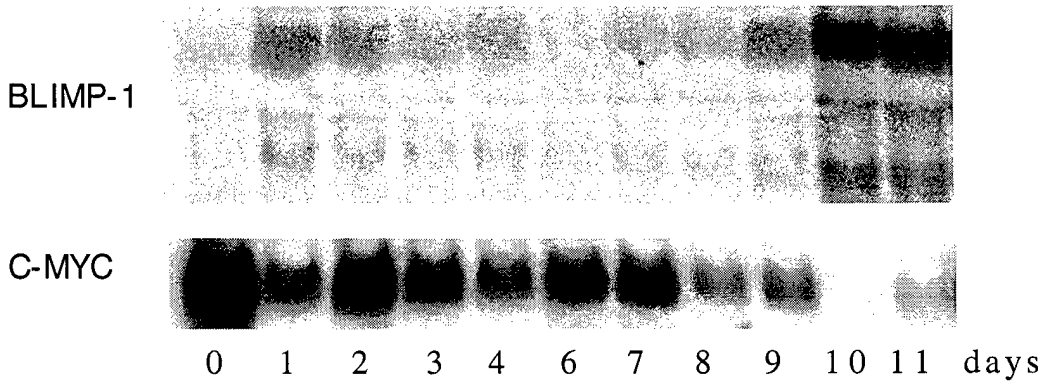


Fig. 1

## C. U937 Cells Treated with PMA

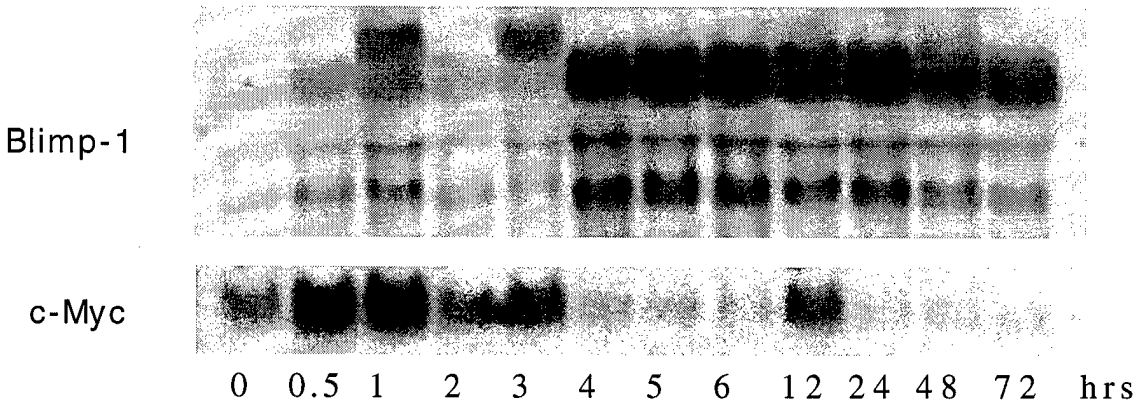


Fig. 2

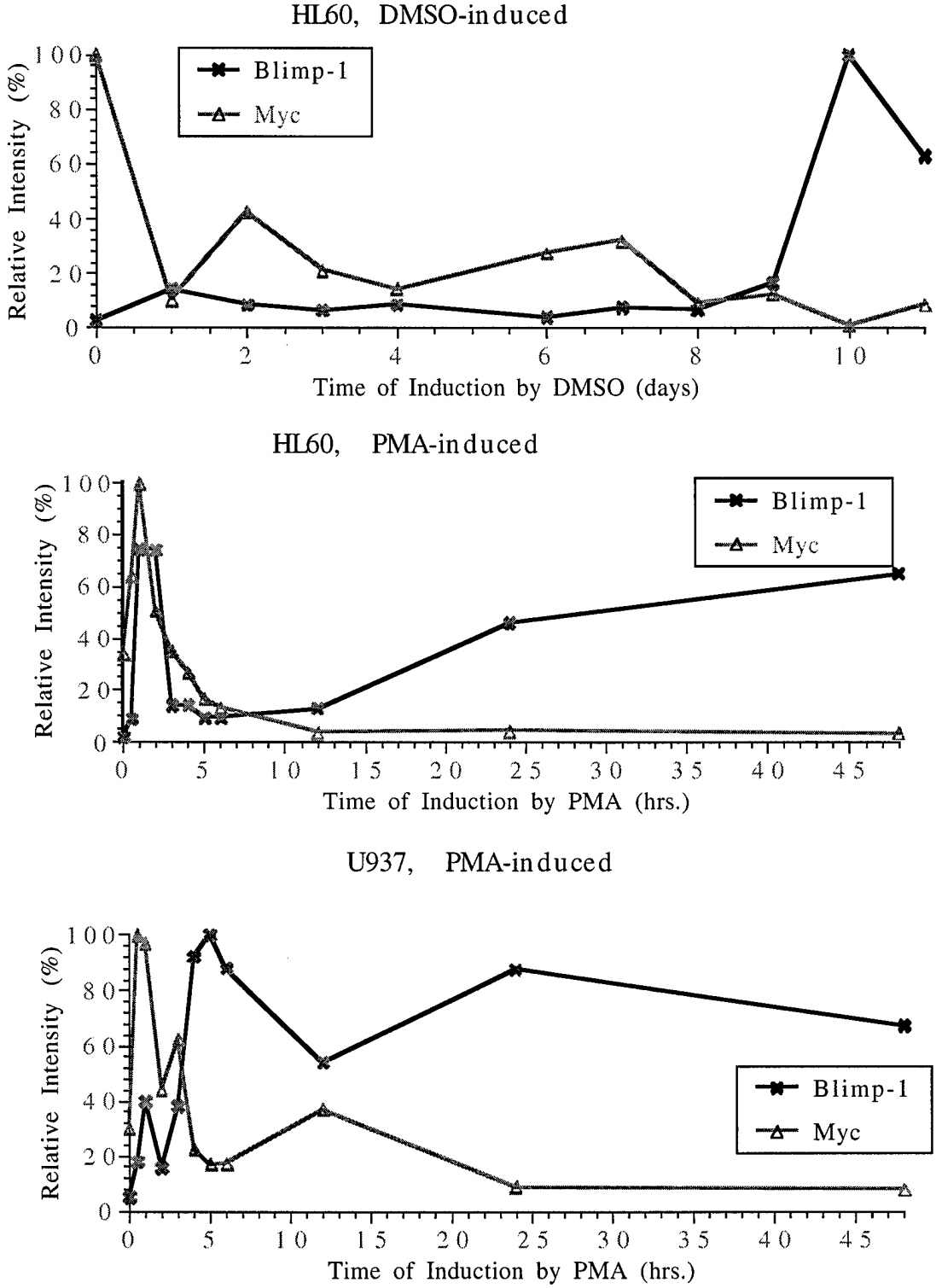

HL60, DMSO-induced
Blimp-1
Myc
Relative Intensity (%)
100
80
60
40
20
0
0
2
4
6
8
10
Time of Induction by DMSO (days)
HL60, PMA-induced
Blimp-1
Myc
Relative Intensity (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
Time of Induction by PMA (hrs.)
U937, PMA-induced
Blimp-1
Myc
Relative Intensity (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
Time of Induction by PMA (hrs.)

Fig. 3

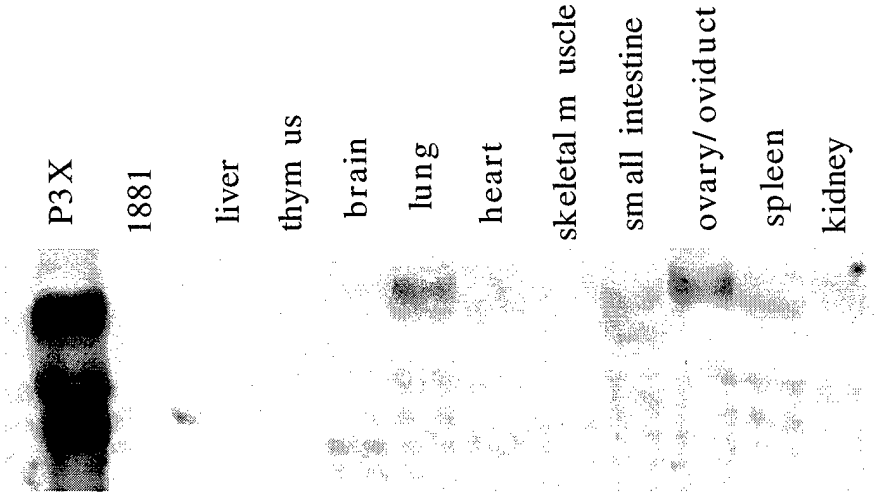
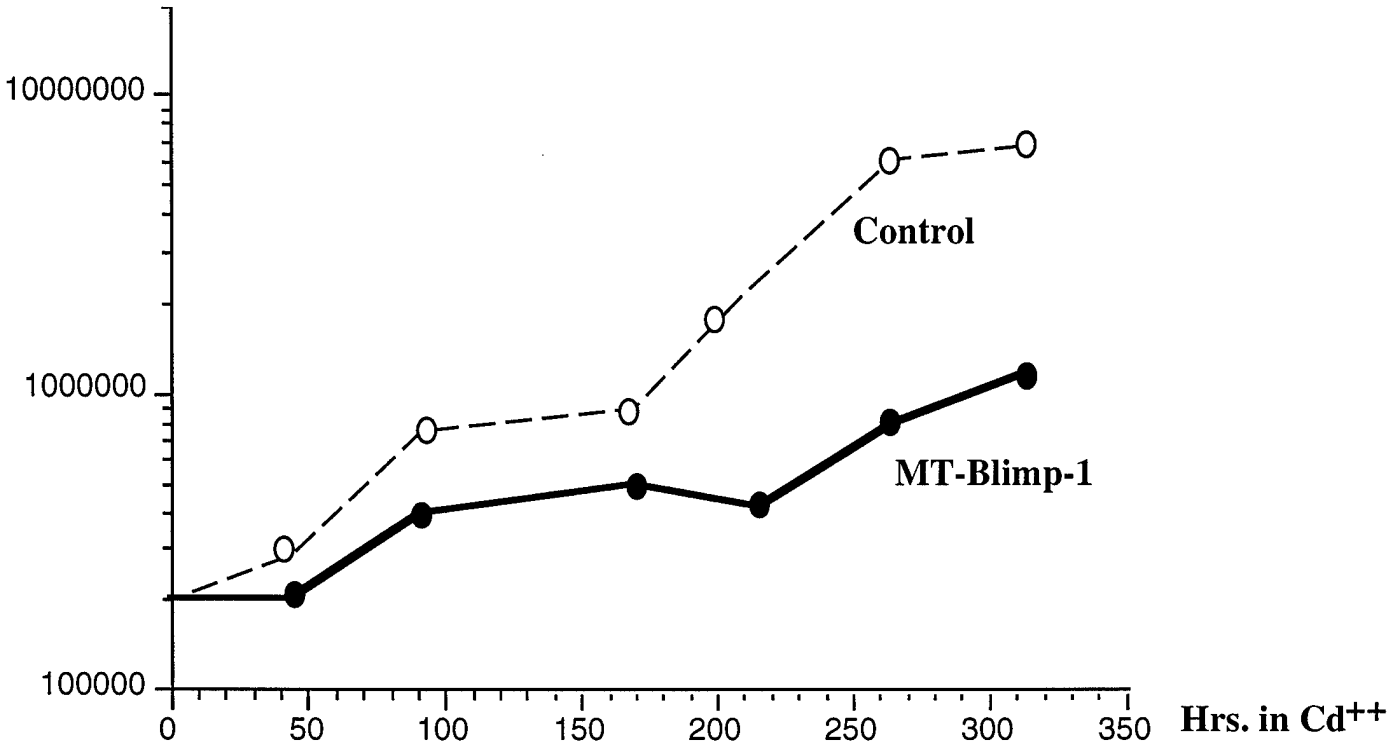

P3X
1881
liver
thym us
brain
lung
heart
skeletal m uscle
sm all intestine
ovary/ oviduct
spleen
kidney

Fig. 4


10000000
1000000
100000
0
50
100
150
200
250
300
350
Control
MT-Blimp-1
Hrs. in Cd++

## CONCLUSIONS

Blimp-1 is a respressor of c-*myc* gene transcription. Our recent work shows that expression of Blimp-1 is not restricted to B lymphocytes, as was previously believed. Thus, Blimp-1 may play an important role in repression of c-*myc* in many tissues. It may also have tumor suppressor activity, since deletions on chromosome 6, where the Blimp-1 gene is located, have been observed in breast cancers and other cancers. In the future, we will determine the expression pattern of Blimp-1 in normal mammary tissue and try to determine its function. We will also actively pursue the possibility that Blimp-1 is a tumor suppressor which may be mutated in some breast cancers.

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## SUBPROJECT II. ESTROGEN-DEPENDENT INDUCTION OF C-MYC TRANSCRIPTION IN BREAST CANCER CELLS

### INTRODUCTION

**Expression of the c-*myc* proto-oncogene is elevated in many breast tumors.** Elevated levels of c-Myc have been found in both estrogen-dependent and estrogen-independent breast tumors (1, 2). In cell lines derived from estrogen-dependent breast tumors, estrogen induces c-*myc* mRNA by a poorly understood mechanism (3-5). In many hormone-independent breast tumors, the c-*myc* gene is amplified, resulting in increased mRNA and protein. However, increased expression of c-*myc* mRNA and protein has also been observed in hormone-independent breast tumors where the gene is not amplified (1, 2). In one case this was shown to be due to increased c-*myc* mRNA stability (3).

Approximately 40% of breast cancer patients have tumors which are estrogen-dependent for growth; however, after anti-estrogen therapy most tumors become estrogen-independent (3). We are particularly interested in understanding the mechanisms which mediate estrogen-inducible c-*myc* expression in estrogen-dependent breast cancer cell lines because this should help us understand important regulatory mechanisms in early breast tumors.

**c-Myc is necessary for growth of breast cancer cell lines and elevated c-Myc is one causal step in malignant transformation of breast tissue.** Antisense c-*myc* oligonucleotides have been shown to inhibit c-*myc* expression and growth of both estrogen-dependent and estrogen-independent breast cancer cell lines (6). This directly establishes that c-Myc protein is required for growth of breast cancer cell lines. A similar requirement for c-Myc function has been demonstrated in other transformed cell lines (7).

Although over-expression of c-Myc alone does not cause malignant transformation of mammary epithelial cells, the elevated levels found in many breast tumors suggest it can be one causal step in transformation (1). This possibility is corroborated by the demonstration that inhibiting c-*myc* expression in breast cancer cell lines inihits their growth (10). It is also corroborated by experiments using mice. Expression of a c-*myc* transgene driven by the murine mammary tumor virus long terminal repeat caused a dramatic increase in murine mammary tumors (8) and expression of the v-*myc* gene caused preneoplastic growth in primary mammary epithelial cells transplanted into mammary fat pads (9).

**The estrogen-dependent expression of c-*myc* has been studied in hormone-dependent breast cancer cell lines.** c-*Myc* mRNA increases rapidly (within one hour) after estrogen stimulation of human breast cancer cells lines which are hormone-dependent. The extent of mRNA induction is 10-12 fold (10). The induction occurs without new protein synthesis (10) and transcripts from both promoter P1 and promoter P2 are increased. Dubik et al demonstrated a rapid increase in transcription initiation by a nuclear run-on transcription assay; however, they used only one, double-stranded probe which precluded any assessment of polymerase pausing or premature termination within exon one and did not distinguish sense and anti-sense transcripts (10). Another study, using a similar probe, in which later times were analyzed failed to show that estrogen increased transcriptional initiation (11). Thus, it seems likely that estrogen affects either initiation or processivity of c-*myc* transcription but additional data are needed to confirm and clarify this important point.

In other genes estrogen induction of transcription initiation has been well-documented (12-14). In these cases, estrogen binds the estrogen receptor (ER), a zinc finger protein which binds to DNA sequences called estrogen response elements (EREs). Binding of ER to EREs confers altered chromatin conformation and increases transcription initiation. Depending on the context, ER sometimes requires interaction with other proteins to mediate its transcriptional effects (15, 16).

Estrogen response elements (EREs) have been sought in the c-*myc* gene but a consensus ERE is not evident. In one study c-*myc*- promoter-dependent reporters were either transfected into MCF-7 cells or cotransfected into HeLa cells with an estrogen receptor expression vector (17). 116 bp surrounding c-*myc* promoter P2 were sufficient for estrogen responsiveness of the reporter in HeLa cells but transcription of the reporter was not inhibited by the antiestrogen tamoxifen even though tamoxifen inhibits transcription of the endogenous c-*myc* gene in MCF-7 cells. Tamoxifen is known to compete

with estrogen for binding to one of the activation domains of the estrogen receptor (ER) (18) and estrogen-dependent effects mediated by the ER should be inhibited by tamoxifen; therefore the transfection study may not adequately reflect normal estrogen activation of c-*myc*. Neither the 116 bp region identified in this study, nor a larger region surrounding the human *c-myc* gene contains a consensus ERE sequence (Merrell and Calame, unpubl.). Thus, the mechanism by which estrogen induces c-*myc* expression in human breast cancer cells is not well-understood and may be complex or unusual compared to other estrogen-inducible genes.

Our studies are focused on determining how estrogen induces c-*myc* mRNA in breast cancer cells. In the initial experiments reported here we have used runon transcription in isolated nuclei to show that estrogen increased transcription initiation rather than transcriptional processivity of c-*myc* in MCF-7 cells. We have also identified four DNase hypersensitive sites in the c-myc gene which are induced in response to estrogen and may be important for mediating the estrogen-dependent induction.

## BODY

### METHODS

*Nuclear runon assay.* MCF-7 cells were grown under + estrogen conditions in IMDM supplemented with 10mg/ml of insulin and 10% FBS. To estrogen-deplete the cells, plates were washed once with PBS at 70% confluency and growth-arrested using phenol red-free IMDM supplemented with 10% charcoal stripped FBS for 48 hours. Transcriptionally active nuclei were isolated by trypsinizing $2x10^8$ MCF-7 cells, washing them twice in PBS, and lysing them in NP-40 lysis buffer Lysed cells were incubated for 5 minutes on ice, the nuclei pelleted by spinning at 200xg in a Beckman Model T-J6 centrifuge and washed once in NP-40 lysis buffer. Nuclear run-on transcription was performed by adding 40µl of 10 mCi/ml [$\alpha$-$^{32}$P]UTP and incubating 45 minutes at 30°C with shaking. To digest DNA and protein, RNase-free DNase I and proteinase K were added to the transcription reaction and incubated for 5 minutes at 30°C and 30 minutes at 42°C, respectively. RNA was extracted with an equal volume of 25:24:1 buffered phenol/chloroform/isoamyl alcohol and TCA-precipitated in the presence of tRNA carrier. The precipitate was filtered onto a 0.45-µm Millipore HA filter, washed, and treated again with DNase I and proteinase K. RNA was eluted from the filter by heating to 65°C for 10 minutes and extracted once more with 25:24:1 phenol/chloroform/isoamyl alcohol, then ethanol precipitated and resuspended in TES/NaCl solution. Hybridization of RNA to cDNA immobilized on a nitrocellulose membrane strip was performed in a scintillation vial for 36 hours at 65°C in a total volume of 1 ml with a probe activity of $10^7$ cpm/ml. Washes were carried out in 2x SSC at 65°C followed by treatment of the filter with RNase A to remove single stranded overhanging ends.

*DNase I hypersensitive site mapping.* $5x10^7$ MCF-7 cells were trypsinized and washed twice with ice cold PBS, then lysed in 3 ml of cold RSB (10 mM Tris pH 7.9, 10 mM NaCl, 5 mM $MgCl_2$) by adding NP-40 to a final concentration of 0.35%. Lysis was allowed to proceed for 5 minutes on ice and checked for completeness under the microscope; if nuclei did not appear free of membranous material, they were gently pipeted up and down and left in lysis soluton on ice for another 5 minutes. Nuclei were pelleted by spinning at 200xg in a Beckman Model T-J6 tabletop centrifuge, resuspended in 2 ml of cold KPS (85 mM KCl, 5 mM Pipes pH 7.5, 5.5% sucrose, 0.5 mM spermidine), and aliquots of 200 ml pipeted into eppendorf tubes for subsequent DNase I digestions. Nuclei were pelleted by spinning at 200xg in an eppendorf microfuge and resuspended in cold DNase I digestion buffer (KPS supplemented with 3 mM $MgCl_2$ and 0.5 mM $CaCl_2$ ) at approximately $5x10^7$ nuclei/ml. DNase digestions were carried out at room temperature for four minutes at DNase I concentrations of 0 - 100 U/ml and stopped by adding four volumes of STOP buffer (12.5 mM EDTA, 0.3125% SDS) and Proteinase K to 50 mg/ml. DNA was incubated overnight at 37°C and purified by two phenol/sevag extractions followed by two sevag extractions and ethanol precipitation. DNA pellets were washed in 70% ethanol and resuspended in 100 ml of TE, and DNA concentrations in each tube calculated from O.D. readings at 260nm. 30 µg of DNA from each tube were digested to completion with a suitable restriction enzyme and hypersensitive sites determined using a standard Southern Blot protocol and a probe hybridizing to either the 5' or 3' end of the restriction fragment of choice.

### RESULTS

**Transcription initiation but not transcriptional processivity of c-*myc* is induced by estrogen treatment of MCF-7 cells.** Runon transcription in isolated nuclei was used to determine polymerase density on the c-*myc* gene. Single-stranded probes were used to ensure that sense but not anti-sense transcripts were studied. Three probes (I, II and III), corresponding to regions +47 to +169 bp, +160 to +349 bp and +775 to +911 bp from the P2 transcription initiation were used (Fig. 5). These probes have been used previously to distinguish transcription initiation and release of paused polymerases within the c-*myc* gene (19). The data in Fig. 6 show that transcription initiation increased three fold in response to estrogen, when polymerase density on Probe I for cells grown in the presence of estrogen is compared to polymerase density on Probe I for cells grown in the absence of estrogen. When processivity of the polymerase was measured by comparing polymerase loading on Probe I to Probe III, no significant difference was observed for cells grown in the presence or absence of estrogen (compare 38.7% minus estrogen and 34.7% plus estrogen). Further experiments are in progress to confirm these results. However, our tentative conclusion is that estrogen induces initiation of transcription of the c-*myc* gene but does not alter the processivity of polymerase once transcription has been initiated. We conclude that increased transcription is responsible, at least in part, for the increased steady-state levels of c-*myc* mRNA when MCF-7 cells are treated with estrogen.

**Identification of estrogen-dependent DNase I hypersensitive sites within the c-*myc* gene.** Having determined that estrogen affects initiation of c-*myc* transcription, we wish to study the mechanism responsible for this effect. As discussed above, there are no consensus EREs within approximately 2 kb 5' or more than 20 kb 3' of the major transcription initiation sites at P1 and P2. Thus, the estrogen-dependent effect may be mediated by a non-consensus ERE or site unrelated to an ERE which binds ER in combination with another protein In order to search the entire c-*myc* gene, both 5' and 3' of the coding region, for site(s) which might be bind activators in an estrogen-dependent manner, we have utilized DNase I hyersensitive site mapping. Fig. 7 shows a map of the c-*myc* gene and indicates the end-labeling probes which have been used for these studies. When these studies are complete we will have searched more than 45 kb 5' of the c-*myc* region, including 6.5 kb 5' and~35 kb 3'of the coding exons. Note that the 3' region includes the region recently identified as a 3' transcriptional enhancer element.

Fig. 8 shows a typical blot from this series of experiments; this is a ScaI digest using probe/digest combination #2 from Fig. 7. In addition to the full-length fragment of 8.5 kb, two smaller fragments (arrows) are seen, indicating DNaseI hypersensitivity sites. When chromatin preparations from MCF-7 cells grown in the absence and presence of estradiol are compared, it is clear that these hypersensitive sites are present in cells grown in estradiol but not in cells grown in the absence of estradiol. Fig. 9. summarizes the combined results of multiple experiments using a variety of probes. The studies on the region including the exons and regions 5' are complete; additional experiments are in progress to finish mapping hypersensitive sites 3' of the gene. The present results show that in MCF-7 cells we observe most of the hypersensitive sites in the vicinity of the three exons which have been reported by others in different cells. Many of the hypersensitive sites were present regardless of whether the MCF-7 cells were quiescent, in the absence of estrogen, or were growing in the presence of estrogen. However, significant differences were observed. Three regions of hypersensitivity were present in the absence of estrogen and disappeared upon treatment with estrogen. The significance of these sites is not clear although in theory they could represent the binding site of a repressor which cannot bind in the presence of estrogen. Four hypersensitive sites appeared when the cells were grown in estrogen. These could correspond to the binding sites for an estrogen-dependent activator.

## DISCUSSION

It has been known for many years that in some breast cancer cell lines such as MCF-7, c-*myc* steady-state mRNA levels increase approximately ten fold upon treatment with estrogen. However, whether or not this increase in steady-state mRNA was caused by increased transcription or by post-transcriptional mechanisms has been controversial. Two groups performed runon transcription analyses; data from one study showed increased transcription while data from the other study showed no increase in transcription (10, 11). One likely explanation for the disagreement in these experiments is that both groups used double-stranded c-myc probes. Subsequent studies have shown that antisense transcription of c-*myc* occurs. Since double-stranded probes measure both sense and anti-sense

transcription, the results of runon experiments using them reflect initiation at multiple promoters and are virtually impossible to interpret. In addition, subsequent studies have shown that, dependent on the probe chosen, runon results can measure transcription initiation and/or polymerase pausing on the c-*myc* gene (19). Therefore, we have performed runon analysis of c-*myc* transcription in MCF-7 cells using three single-stranded probes, which allow is to distinguish between effects on transcription initiation and polymerase processivity. Our results show that in MCF-7 cells, estrogen primarily increases c-*myc* transcription initiation. This is consistent with the classical effect of steroid hormones, which have been shown to activate transcription of many genes upon binding to their cognate receptor (12, 13). This information is important in order to further study the estrogen-dependent mechanism. However, it is important to note that the 2-3 fold increase in transcription measured by runon analysis does not entirely account for the >10 fold increase in steady-state mRNA. This may simply reflect technical limitations of the runon experiments or it may be that post-transcriptional mechanisms also contribute to the overall increase in c-*myc* mRNA.

Since no consensus ERE is present in nearly 30 kb surrounding the c-*myc* gene, sequence analysis is not informative in determining how estrogen induces c-*myc* transcription. Binding sites for proteins which activate or repress transcription are usually found to be hypersensitive to DNase I when chromatin is treated with nuclease. Although not all DNase I hypersensitive sites are regulatory elements, most regulatory elements are hypersensitive sites. Thus, we reasoned that a thorough analysis of estrogen-dependent hypersensitive sites in the c-*myc* gene should help us identify the site(s) required for the estrogen-dependent induction.

Our data have identified four estrogen-induced hypersensitive sites and three hypersensitive sites which disappear upon estrogen treatment. We plan to study the estrogen-induced hypersensitive sites first. We reason that the simplest model for how estrogen induces transcription is that it recruits a transcriptional activator, probably a protein complex which includes estrogen bound to ER. One or more of these hypersensitive sites may be the binding site for such an activator. Site I corresponds to the two major transcriptional initiation sites, P1 and P2. Thus, it is unlikely to contain the activator binding site. Therefore, sites II, III and IV are the regions we will study further. We are particularly intrigued with sites III and IV which occur within a large region which has recently been shown to be a transcriptional enhancer for the c-*myc* gene.

## FIGURE LEGENDS

Fig. 5. Map of the c-*myc* gene showing the regions used for single-stranded probes in the runon assay.

Fig. 6. Polymerase loading on the c-*myc* gene in MCF-7 cells grown in the presence and absence of estrogen. The amount of radiolabeled RNA which hybridized to each probe on the filter was quantitated using a PhosphoImager, normalized to the GAPDH control and corrected for the number of U's in the region.

Fig 7. Strategy for mapping hypersensitive sites within the c-*myc* gene. The top line represents the genomic region containing the c-myc gene and its surrounding regions. The open box indicates the 5' untranslated exon which contains the two major transcription initiation sites, P1 and P2. The hatched boxes indicate the coding exons. The solid boxes beneath the gene indicate end-labeling probes which were used with restriction digests which allowed hypersensitive sites within the regions indicated by the arrows extending from the boxes to be mapped. Each region of the c-*myc* gene was scanned using more than one digest.

Fig 8. Representative blot showing DNase I hypersensitive sites using a ScaI digest (# 2 in Fig. 7).

Fig. 9. Summary of DNase I hypersensitive sites found in the c-*myc* gene in MCF-7 cells. The c-myc genomic region is represented as described in Fig. 7. DNase I hypersensitive sites which appeared consitiutively, only in the absence of estrogen or only in the presence of estrogen are indicated by arrows. The estrogen-induced hypersensitive sites are also indicated by roman numerals.

Fig. 5

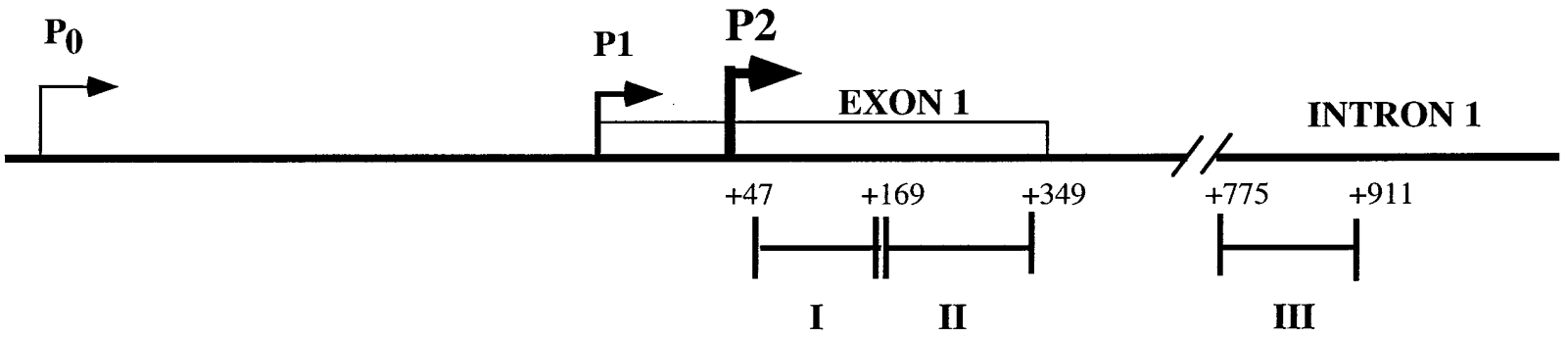


Fig. 6

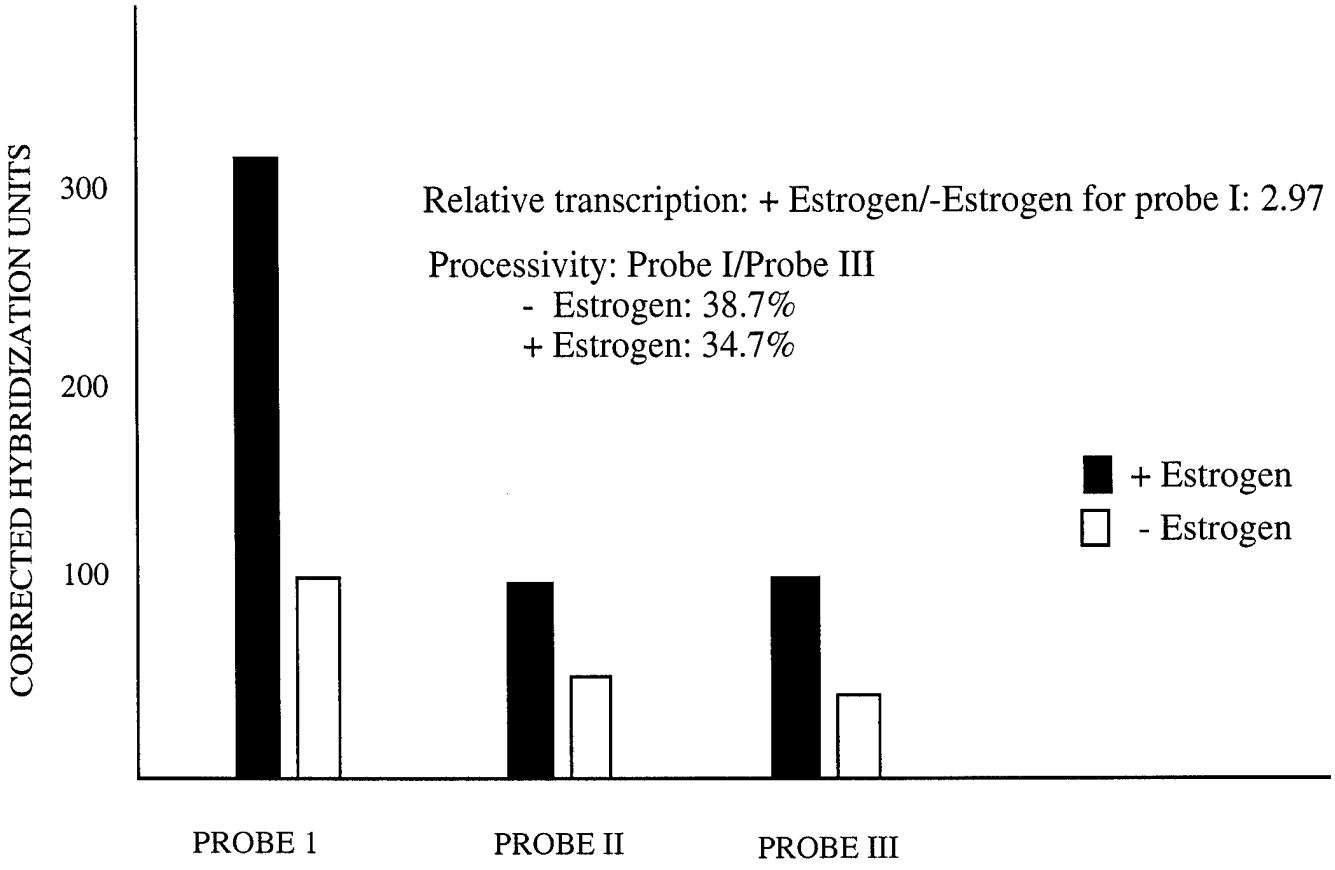
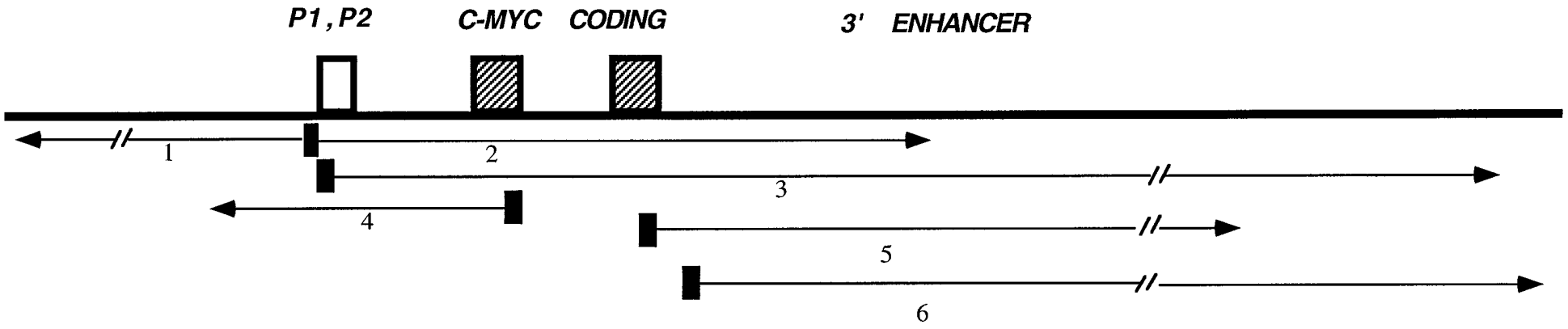

CORRECTED HYBRIDIZATION UNITS
300
200
100
Relative transcription: + Estrogen/-Estrogen for probe I: 2.97
Processivity: Probe I/Probe III
- Estrogen: 38.7%
+ Estrogen: 34.7%
+ Estrogen
- Estrogen
PROBE 1
PROBE II
PROBE III

Fig. 7

## End-labeling Probes Used for Hypersensitive Site Mapping




1 Xho digest from -7000 to +63 bp
2 Sca I digest -295 to +8272 bp
3 Xho I digest from +67 to +20,299 bp
4 BglII digest from -3,200 to +3,058 bp
5 Apa I digest from +4033 to 16, 103 bp
6 Eco R1 digest from +5755 to 21, 352bp

Fig. 8

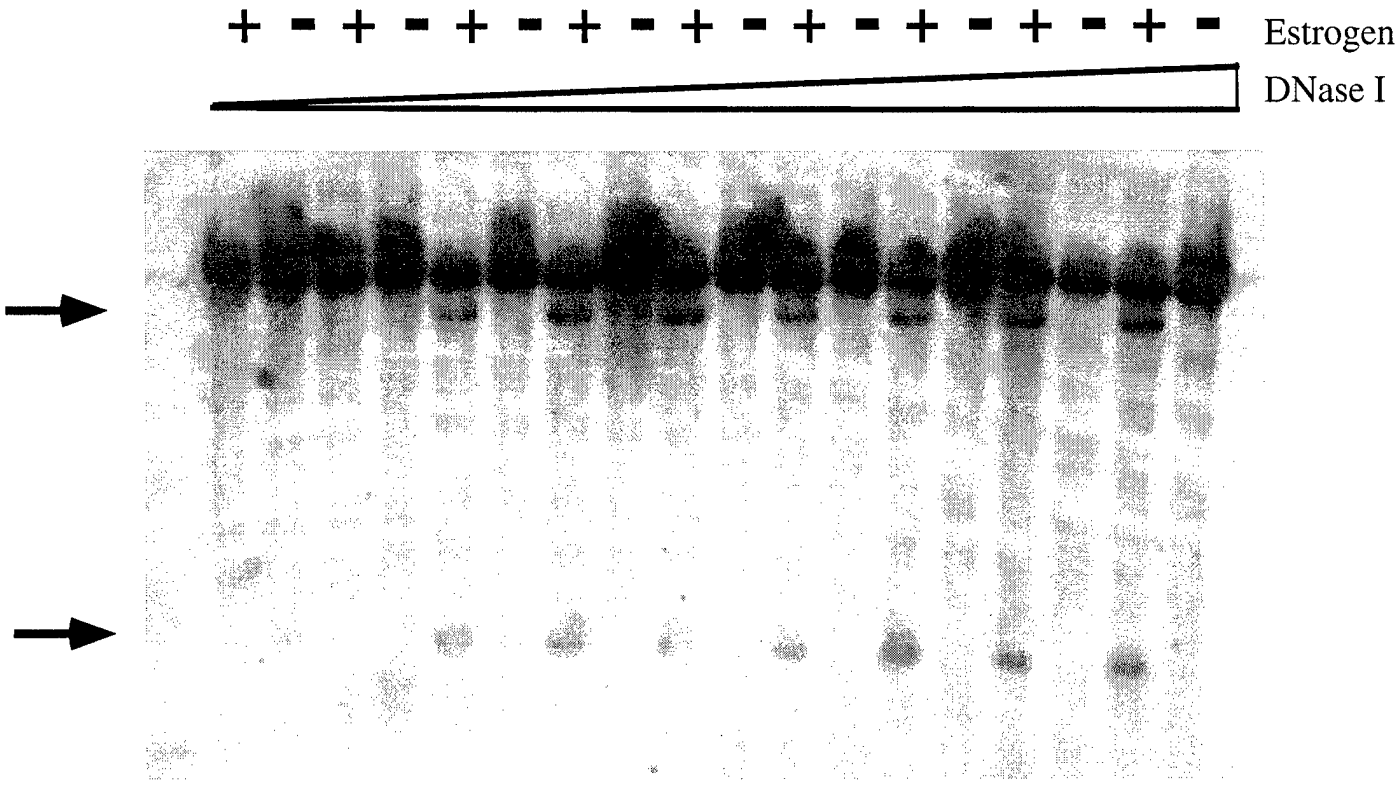
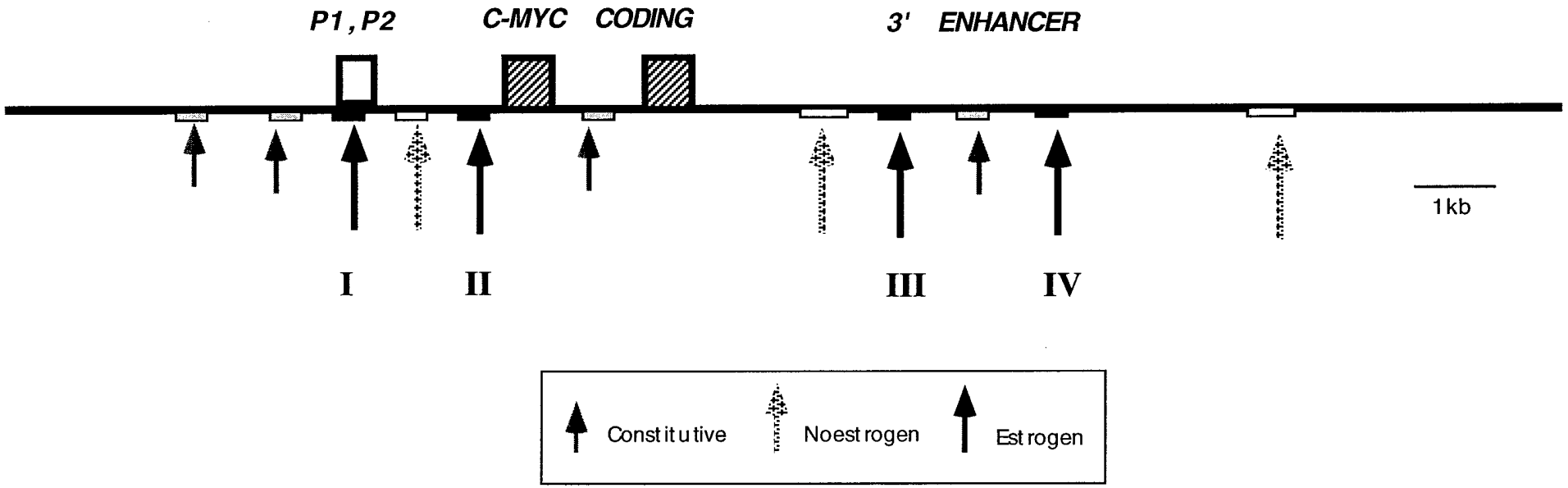


Fig. 9

## DNase I Hypersensitive Sites in the c-*Myc* Gene in MCF-7 Cells



## CONCLUSIONS

The current data are consistent with the idea that estrogen induces c-*myc* transcription by a non-classical mechanism--i.e. not using normal ERE sites. The three estrogen-induced DNaseI hypersensitive sites which we have identified downstream of P1 and P2 are good candidates for regions which mediate this estrogen-dependent induction. Having identified them, we will now test their activity and determine what proteins bind to them.

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